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13. ABSTRACT *(Maximum 200 Words)*

This study set out to evaluate the biological consequences of a shift in the ratio of the serine protease matriptase relative to its cognate inhibitor HAI-1. The goal of this work is to generate data that we believe will assist in evaluating whether the matriptase HAI-1 system plays a role in breast cancer invasion and metastasis. In order to alter protease / inhibitor ratios we proposed to use a tetracycline regulable expression system to either increase matriptase levels by overexpression or reduce HAI-1 levels by expression of a ribozyme targeted to the HAI-1 mRNA. Although we have encountered significant technical difficulties with this project, we have made considerable progress. Having obtained a full length cDNA clone of matriptase we generated cell lines that over express the gene in an inducible fashion and have started to evaluate the biological consequences of such overexpression. Thus far we have found that such overexpression has no effect on cell proliferation or on the synthesis or activation of two matrix metallo proteases – MMP-2 and MMP-9. Work is rapidly proceeding on the remaining studies.

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Introduction:

Matriptase is a serine protease that is made my epithelial cells in many tissues including the mammary gland. In tissue culture systems and normal tissue, it is always co-expressed with its cognate inhibitor KSIP-1, which we now refer to as HAI-1 (Hepatocyte Activator Inhibitor-1). In breast tumors, it appears that this association between the two proteins is somewhat loser and so this project set out to characterize the biological implications of relative over expression of matriptase versus HAI-1. The goal of the research is to provide data that will assist in the evaluation of HAI-1 and matriptase determinations as prognostic markers and matriptase as a target for therapy.

Body:

Progress on Task 1.

In order to study the implications of altered matriptase – HAI-1 ratios on breast cancer cell biology, this project set out to directly alter the levels of these proteins in the breast cancer cell line MCF-7 and then to characterize any changes in the behavior of the cells. The method that we chose to achieve this goal was to use the tetracycline-inducible expression system. We had recently cloned what we believed was a full length clone for matriptase and so proposed to use this cDNA in the tetracycline to inducibly overexpress matriptase thereby increasing the matriptase to HAI-1 ratio. We had obtained MCF-7 cells that were already transfected with the tetracycline responsive transactivator protein from Dr Yee in Minnesota and planned to transfect these cells with the cDNA under the control of a tetracycline responsive promoter, by co-transfection with the plasmid pCHC6-βgal which confers resistance to hygromycin and directs the expression of the bacterial gene betagalactosidase.

However, shortly after the commencement of the project, inspection of the sequence that we had cloned, and comparison with the sequence of the mouse homolog of the matriptase (epathin), lead us to the realization of the fact that our clone was missing a portion of the five prime end. Thus, the first goal of the project became the cloning of the true 5' prime end of the cDNA as a prerequisite for the preparation of the expression constructs needed for the project. As described below, this proved to be a somewhat difficult process which we now understand was in part because in common with an increasing number of genes, the matriptase gene has an extremely large first intron. The analysis of the genomic structure of the matriptase gene was an important part of this work and the structure is presented in figure 1 and table 1. The size of the first intron meant that genomic approaches to cloning the 5' end of the gene were destined to be unproductive.

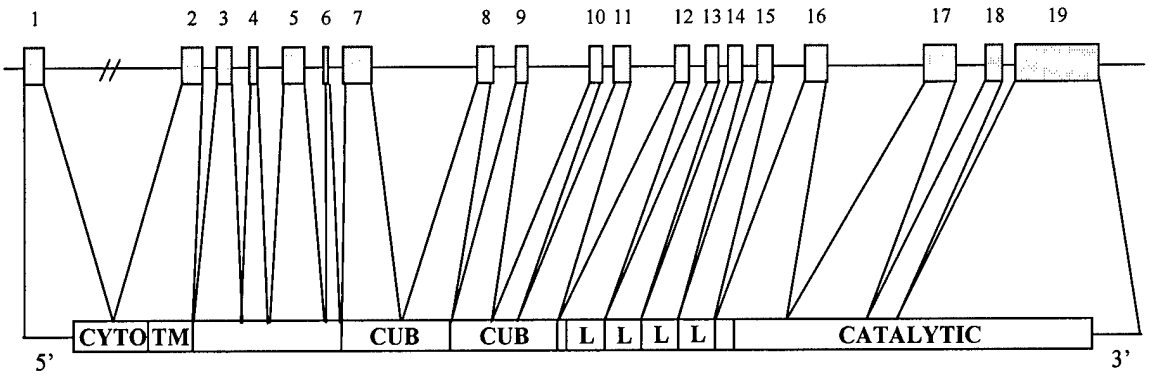

Figure 1. Schematic structure of the matriptase genomic structure (top) in relation to the matriptase cDNA (bottom), not drawn to scale. Numbers indicate sequence of exons. CYTO, cytoplasmic domain; TM, transmembrane domain; CUB, C1r/s, Uegf, and Bone morphogenic protein-1 domain; L, low density lipoprotein receptor (LDLR)-like domain; CATALYTIC, serine protease catalytic domain.

Table 1

Exon	Size	3′ Exon Junction	Intron	5′ Exon Junction	Exon	Junction type
		Arg His Glu[27]	28054 bp	[28]*Lys Val Asn*		
1	?	CGG CAC GAG	GTGAGCGC---CCCTGCAG	AAA GTG AAT	2	0
		Leu Gln T[81]	257 bp	[81]*yr Arg Asp*		
2	160	TTG CAG T	GTGAGTAA---TCACACAG	AC CGG GAC	3	I
		Lys Asp Ala[123]	212 bp	[123]Leu Lys Leu		
3	128	AAG GAC GCG	GTGAGTGC---TTCCTCAG	CTG AAG CTG	4	0
		Ala Phe Se[147]	800 bp	[147]r Glu Gly		
4	71	GCC TTC AG	GTGGGTGT---CTCCCCAG	C GAG GGC	5	II
		Ala Phe P[200]	252 bp	[200]*ro Thr Asp*		
5	158	GCT TTC C	GTGAGTTC---TTCTTCAG	CC ACG GAC	6	I
		Gln Asp A[212]	306 bp	[212]*sn Ser Cys*		
6	36	CAG GAC A	GTAAGTAT---CTGCGCAG	AC AGC TGC	7	I
		Leu Val Gl[292]	3453 bp	[292]*n Leu Cys*		
7	241	CTG GTG CA	GTGAGTAC---CCCCACAG	G TTG TGT	8	II
		Met Ser S[339]	352 bp	[339]*er Cys Gly*		
8	140	ATG AGC A	GTAAGGAA---CCTCTCAG	GC TGT GGA	9	I
		Asn Ile Glu[371]	1601 bp	[372]Val Pro Asn		
9	98	AAC ATT GAG	GTAGGAGC---TCCCTCAG	GTG CCC AAC	10	0
		Gly Glu Ly[408]	122 bp	[408]*s Tyr Cys*		
10	110	GGG GAG AA	GTGAGTCC---CCCTCCAG	A TAC TGC	11	II
		Ser Asp P[452]	1142 bp	[452]ro Cys Pro		
11	131	AGT GAC C	GTGAGTGA---GCCCGCAG	CA TGC CCG	12	I
		Asn Cys S[487]	363 bp	[487]*er Cys Asp*		
12	105	AAC TGC A	GTGAGTCA---CACTCCAG	GT TGC GAC	13	I

Exon	Size	3′ Exon Junction	Intron	5′ Exon Junction	Exon	Junction type
		Gly Cys S[524]	100 bp	[524]*er Cys Pro*		
13	111	GGG TGC A	GTGAGTGC---CTCTCCAG	GT TGT CCG	14	I
		Pro Lys V[562]	326 bp	[562]*al Asn Val*		
14	114	CCC AAG G	GTGAGGCC---TTGAACAG	TG AAC GTC	15	I
		Asp Cys A[603]	882 bp	[603]*sp Cys Gly*		
15	123	GAC TGC G	GTGAGCAG---CTCTGCAG	AC TGT GGG	16	I
		Gly Phe Ar[665]	8270 bp	[665]*g Tyr Ser*		
16	187	GGA TTC AG	GTGGGTCT---CCTACCAG	G TAC TCA	17	II
		Tyr Gly G[757]	758 bp	[757]*ly Thr Gly*		
17	275	TAT GGA G	GTAAGCTT---CTCCCCAG	GC ACT GGC	18	I
		Ser Cys Gln[802]	84 bp	[803]*Gly Asp Ser*		
18	137	TCC TGC CAG	GTGGCCCC---CCGCCCAG	GGT GAT TCC	19	0
19	697					

Table 1. Exon/intron organization of the matriptase gene. The exon/intron boundaries are assigned according to the GT-AG rule for splice junctions. The size of the first exon is unknown because 5' cloning of the cDNA is not definitive. 0, I, and II indicate the position of introns between coding exons: 0, interuption occurs between the codons; I, interuption occurs between the first and second base; II, interuption between the second and third base.

Our first attempts to clone the 5' end of the gene were by 5' RACE of the matriptase cDNA. However, this procedure met with only limited success, probably due to a highly GC-rich area in the matriptase 5' coding region that prevented obtaining clones longer that a cDNA about 50 base-pairs short of the full-length clone. To circumvent this problem, we designed primers based upon the epithin 5' untranslated region, and used these in a "touch-down" PCR protocol designed to give a specific PCR product despite mismatches in primer-template base-pairing as described below.

The cDNA template for 5' cloning of matriptase was obtained from a Marathon-Ready human fetal kidney cDNA 5' RACE kit purchased from Clontech (Clontech, Palo Alto, CA). Primers were designed in the known matriptase 5' end (reverse primer) and in the 5' untranslated region of epithin (forward primers). The sequence of the reverse primer was 5'-CAAGAGATCCTCGTGTACTCAGTAGG—3', and the sequence of the four forward (epithin) primers were 5'-CCGGAACTTGGCCCGTGCCTCACCT-3', 5'-TCGTCCCTCGTGTCCCCAACACCCC-3', 5'-ATGGGTAGACGGCTGCCCGGAGGGAC-3', and 5'-TGCCTCACCTCGTCCCCCTCGTCC-3'. The PCR protocol used a "touch-down" procedure, and had the following parameters: initial denaturing for 1 cycle at 95°C for 1min30sec, 5 cycles of denaturing at 95°C for 20sec/annealing and elongation at 74°C for 2min, 5 cycle of denaturing at 95°C for 20sec/annealing and elongation at 72°C for 2min, and 25 cycles of denaturing at 95°C for 20sec/annealing and elongation at 70°C for 2min, followed by a terminal elongation for 1 cycle at 68°C for 7min. The vectors used for cloning included the pCR2.1 TA cloning vector and the pcDNA3.1 mammalian cloning vector from Invitrogen (Invitrogen, Carlsbad, CA), and the pUHD10-3 tetracycline-inducible expression vector from Dr H. Bujard. Cos7 cells were obtained from the Lombardi Cancer Center Tissue Culture Shared Resource, and were transfected using lipofectamine transfection reagent purchased from Gibco LifeTechnologies (Gibco, Rockville, MD). Restriction enzymes were purchased from Gibco and New England Biolabs (NEB, Beverly, MA).

Only one of the epithin-based forward primers was successful in generating a PCR product. This PCR product was cloned into the pCR2.1 TA cloning vector according to the manufacturer's protocol using the TA cloning kit from Invitrogen. Sequencing of the PCR product revealed that it contained the mouse epithin-based primer and the downstream human matriptase 5' untranslated region and 5' coding region. Luckily, the first 5' stop codon within the 5' untranslated region of matriptase was found, indicating that the further cloning of the matriptase 5' end was unnecessary for the purposes of obtaining a full-length matriptase clone. To derive a full-length clone, a piece of DNA representing the 5' end of matriptase was cut out of the pCR2.1 TA vector using BamHI and BstXI restriction enzymes, and sub-cloned into a pcDNA3.1 vector containing the corresponding 3' sequence of matriptase by cutting this vector with BamHI and BstXI. The resulting vector, termed pcDNA3.1-Matriptase, contained the full-length sequence of matriptase. The proper expression of matriptase was verified by transfection of COS7

cells with the pcDNA3.1-Matriptase vector using lipofectamine transfection reagent, followed by western blotting of whole cell lysates using the matriptase-specific monoclonal antibody M32 according to a previously published protocol (Lin, et al., 1997). The full-length sequence of matriptase was then sub-cloned into the pUHD10-3 tetracycline inducible vector by inserting an EcoRI fragment containing the full-length matriptase sequence into an EcoRI-cut pUHD10-3 vector.

Having generated and validated the new full-length expression construct, we proceeded to transfect MCF-7 cells that already contained the tetracycline responsive transactivator protein (described in the orrigional grant proposal) using lipofectamine transfection reagent.

The pUHD10-3-Matriptase construct was co-transfected with the plasmid PCHC6 and transfectants were selected based on their resistance to hygromycin (150 ug/ml, Calbiochem, San Diego CA). The resultant colonies were screened for the expression of Bgal (figure 2) and then colonies were analyzed for tetracycline regulable expression of matriptase (figure 3). Clones that seemed to be functioning correctly were subjected to further analysis to examine the time course of induction (figure 4) and to determine if induction lead to an increase in matriptase activity (m69 staining figure 4).

Figure 2: β-galactosidase staining of transfectants

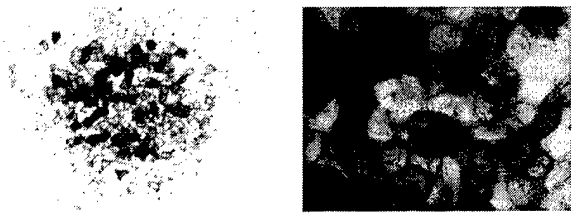

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Figure 3

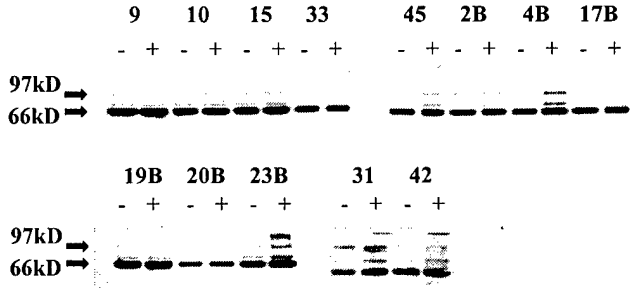
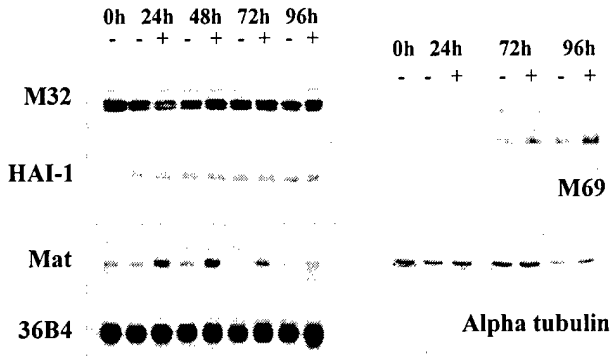
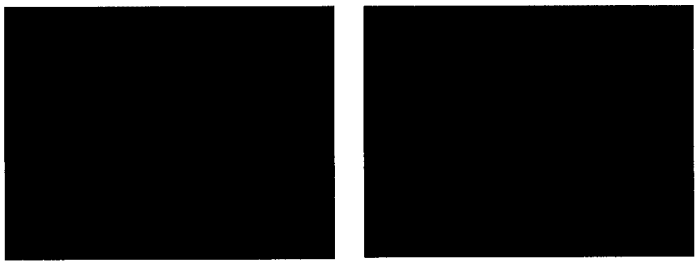


Figure 4



As can be seen from these data we have only been able to achieve very modest increases in matriptase levels in these clones and furthermore, we have found that the these clones are rather unstable and that expression is lost over time (figure 5) and so we have had to sub-clone the expressing lines in order to get more homogeneously expressing lines. overexpression of matriptase, and that the slightly leaky nature of the tetracycline expression system results in sufficient overexpression, that during the time required to select transfectants, highly positive clones are lost.

Figure 5. Relatively few cells overexpress matriptase

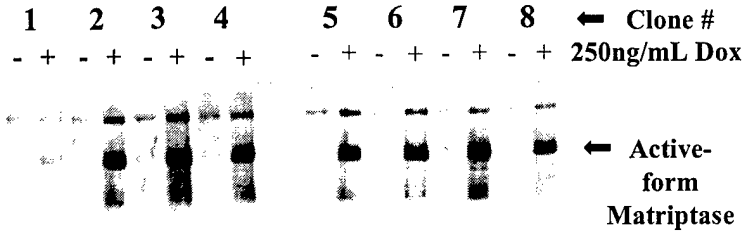


72h - Dox **72h +Dox**

By subcloning the transfectants and expanding the clones rapidly we were able to generate populations of cells in which the level of matriptase expression was widely regulable by tetracycline (figure 6) and where almost all of the cells were expressing the protein (figure 7). These cells were then used in a series of experiments to evaluate the effects of matriptase overexpression.

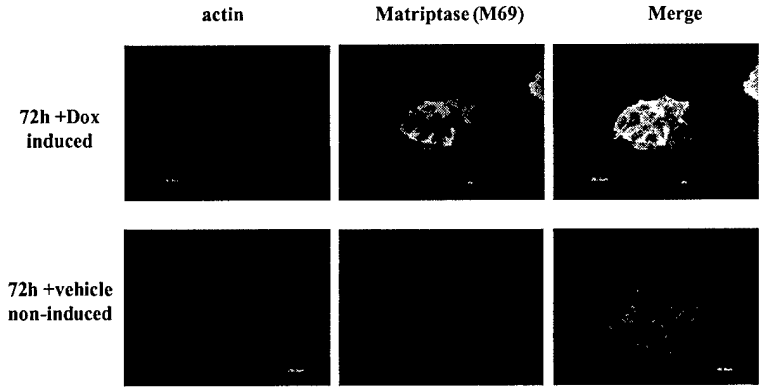
Figure 6

Subcloning Matriptase clone 23B



M69 western blotting

Figure 7 **Anti- active form Matriptase (M69) immunofluorescense**



First the overexpression of matriptase on cells proliferation was evaluated in growth assays in which the number of cells was evaluated by chrystal violet binding (figure 8), and by flow-cytometric analysis (figure 9). As can be seen matriptase overexpression produced no effect on the growth of these cells – the figures are representative examples of several assays conducted with all of the clones generated.

Figure 8

Growth of Matriptase-inducible Clone 23B-3 on Tissue Culture Plastic

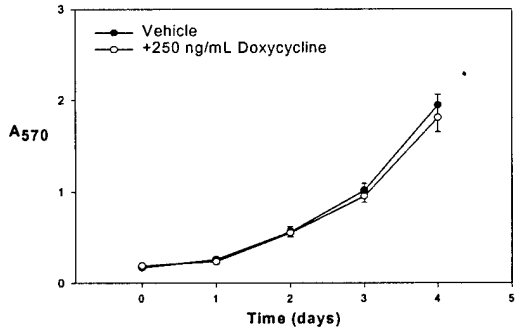
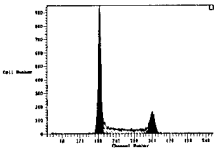
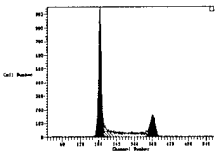


Figure 9

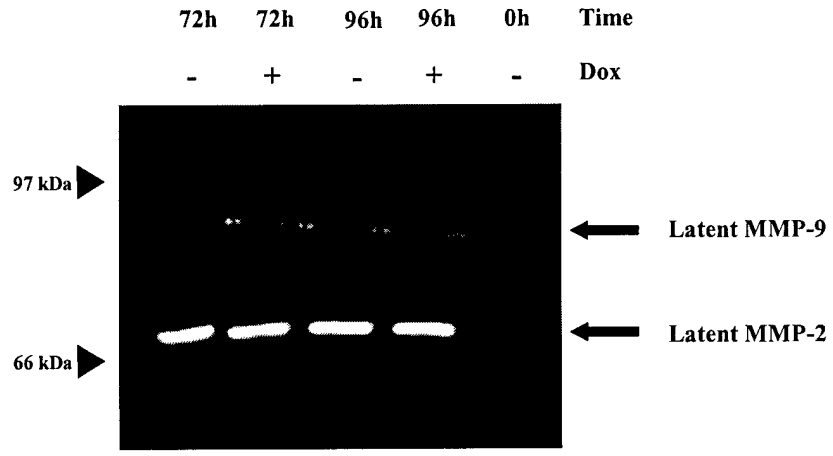
Vindelov Cell Cycle Analysis
Clone 23B-3

		48h	72h	
Vehicle Treated	G0/G1	60.32	54.67	
	G2/M	14.35	19.53	
	S	25.33	25.80	
+250ng/mL Doxycycline	G0/G1	60.85	54.67	
	G2/M	14.35	17.87	
	S	24.79	23.06	

Next we set out to determine if the overexpression of matriptase resulted in any change in the levels of secretion or activation of two other proteases that might be substrates for matriptase – the matrix metallo proteases 2 and 9 (MMPs -2 and 9) . The expression level and activation state of the MMPs was determined by the use of the standard technique of gelatin zymography (figure 10). As can be seen from this figure, the level of MMP expression is not altered and all of the protein seen is in the latent pro-form, which is normal for MCF-7 cells. Thus the increased expression of Matriptase does not seem to have altered the level of MMP expression or activation.

Figure 10

MMP Gelatin Zymography: Clone 23B-3



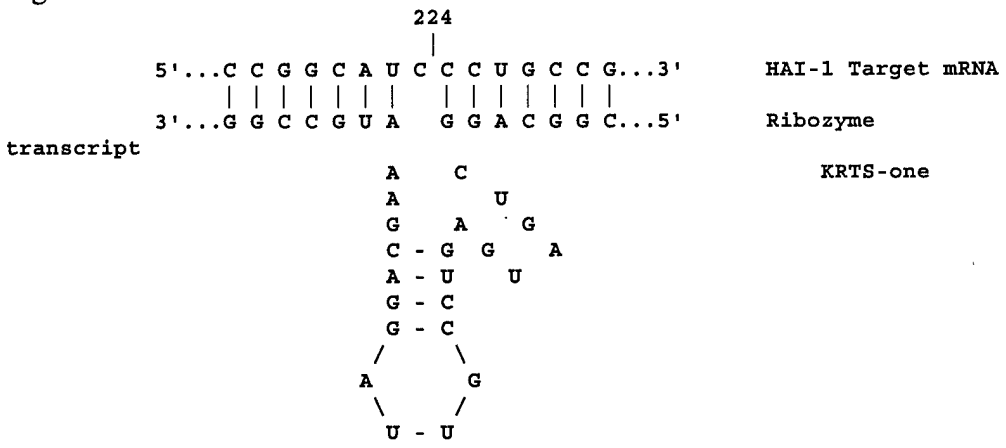
Further studies to evaluate the behavior of these clones are ongoing, and we are in the process of producing additional clones because we have encountered a problem using the existing ones in our animal studies. We have used the cells that we have generated thus

far in a series of animal experiments in which we tried to evaluate the effects of matriptase overexpression on tumor growth and metastasis. However, we have discovered that for reasons that are at this time unclear, the cells will not form tumors in nude mice. We have tried inoculating very high numbers of tumor cells, and have tried mixing the cells with the extracellular matrix preparation Matrigel which is a technique widely used to assist cells form tumors in nude mice. All of these attempts have been unsucessfull. We are in the process of trying to determine why this should be and in the meantime are preparing additional clones using a different parental MCF-7 sub-line.

Progress on Task 2

The other way that we have set out to alter the matriptase / HAI-1 ratio in MCF-7 cells is to reduce HAI-1 expression by the use of the inducible expression of hammerhead ribozymes directed against the HAI-1 mRNA. Ribozymes are catalytic RNA molecules that have homology arms that are targeted against the mRNA that one is trying to down-regulate. Our consultant on this work Dr Anton Wellstein, assisted us in the design of several series of anti-matriptase ribozymes targeted against various parts of the HAI-1 mRNA. Figure 11 shows an example of one of these series.

Figure 11.



		Cleaves after:
KRTS-one	5'..CCGGCATTTCGTCCTAACGGACTCATCAGCCTGCCG..3' 3'..GGCCGTAAAGCAGGATTGCCTGAGTAGTCGGACGGC..5'	224
KRTS-four	5'..GGCACCTTTCGTCCTAACGGACTCATCAGCCTGTTC..3' 3'..CCGTGGAAAGCAGGATTGCCTGAGTAGTCGGACAAG..5'	826
KRTS-seven	5'..GCGGCATTTCGTCCTAACGGACTCATCAGTCCAAGA..3' 3'..CGCCGTAAAGCAGGATTGCCTGAGTAGTCAGGTTCT..5'	1457
KRTS-nine	5'..AGCCCCTTTCGTCCTAACGGACTCATCAGTTGGAGA..3' 3'..TCGGGGAAAGCAGGATTGCCTGAGTAGTCAACCTCT..5'	1854

Single stranded DNA corresponding to the proposed ribozymes were synthesized by LifeTechnologies (Gibco BRL, Rockville, MD), and annealed, phosphorylated, and subcloned into the pcDNA3.1 cloning vector using standard protocols. After verification of the correct ribozyme sequence, all of the ribozymes were sub-cloned into the pUHD10-3 vector, and some of the ribozymes were sub-cloned into into the pBi-tet vector.

To date, we have synthesized six ribozymes that target HAI-1. These six ribozymes cover the length of the HAI-1 coding sequence, including the 5' and 3' untranslated regions and the largest exon present in the cDNA. All six of the HAI-1 targeting ribozymes have been cloned into the pcDNA3.1 mammalian expression vector, to generate pcDNA3.1-HAI-1-ribozyme vectors. In addition, three have been cloned into the tetracycline-inducible, bi-directional GFP expression vector that allows simultaneous expression of the ribozyme and GFP for tracking of transfected cells. These vectors have been termed pBi-tet-GFP-HAI-1-ribozyme vectors. The pcDNA-HAI-1-ribozyme vectors (co-tranfected with a GFP expression vector) and the pBi-tet-GFP-HAI-1-ribozyme vectors have been tested *in vitro* by transient transfection. In this setting, GFP-positive cells are examined for a reduction in HAI-1 expression, as assessed by immunofluorescence staining for HAI-1 using the HAI-1 specific M19 monoclonal antibody. Unfortunately, the ribozyme-transfected cells (GFP-positive) show little evidence of a reduction in HAI-1 protein expression. In addition, the HAI-1 targeting ribozymes have been sub-cloned into the pUHD10-3 tetracycline-inducible expression vector for the inducible expression of HAI-1 ribozymes in stably transfected MCF-7 rTA cells. However, despite considerable effort we have been unable to successfully generate any inducible HAI-1 ribozyme targeting cell lines in which the protein expression of HAI-1 can be inducibly reduced by the addition of doxycycline that turns on the expression of the ribozymes. This has been extensively analyzed by both northern and western blot analysis using the M19 mAb.

We have become somewhat frustrated with our experience using ribozymes to try and reduce HAI-1 levels and so have recently gone to our backup strategy of using antisense constructs instead. We are in the process of constructing several antisense constructs which target various parts of the HAI-1 message and will start testing them as soon as they are ready.

Key Research Accomplishments:

1) We have determined the structure of the matriptase gene locus
2) We have cloned the full 5' prime end of the matriptase cDNA
3) We have generated MCF-7 cell clones which inducibly overexpress matriptase
4) We have shown that such overexpression has no effect on cell growth
5) We have shown that this overexpression does not alter MMP-2 or -9 expression or activation

Reportable Outcomes:

Oberst, M., Anders, J., Xie, B., Singh,B., Ossandon, M., Johnson, M., Dickson, R.B. and Lin, C-Y. (2001) Matriptase and HAI-1 are expressed by normal and malignant epithelial cells *in vitro* and *in vivo*. Am. J. Path. 158:1301-1311.

Oberst, M.D, Lin, C-Y., Dickson, R.B. and Johnson, M.D. (2000) Role of proteases in breast cancer. J. Women's Cancer. 2:201-16

Conclusions:

In conclusion, despite several frustrating scientific difficulties we have made significant progress towards the completion of this study and have learned a great deal about the biology of the matriptase HAI-1 system. As the work proceeds we will rapidly learn more. This is important, since it is clear that this system plays a significant role in mammary gland development and probably carcinogenesis and tumor progression. The technical difficulties have slowed our progress, however, and for that reason and due to some delays in replacing the technician working on the project, we requested and were granted a "no cost extension" increasing the period of the grant by one year. This will provide us with ample time to overcome the technical issues we have encountered and complete the project.

References:

Lin, C.Y., Wang, J.K., Torri, J., Dou, L., Sang, Q.X.A., Dickson, R.B. Characterization of a novel, membrane-bound, 80-kDa matrix-degrading protease from human breast cancer cells. J Biol Chem 272:9147-9152, 1997.

Abbreviations:

βgal	Betagalactosidase
HAI-1	Hepatocyte Activator Inhibitor-1
MMP	Matrix Metallo Protease